NEOPLASIA
www.neoplasia.com

Volume 16 Number 1 January 2014 pp. 84–96 84

Intermittent Metronomic Drug Schedule Is Essential for Activating Antitumor Innate Immunity and Tumor Xenograft Regression[1,2]

Chong-Sheng Chen[3], Joshua C. Doloff[3] and David J. Waxman

Division of Cell and Molecular Biology, Department of Biology, Boston University, Boston, MA

Abstract

Metronomic chemotherapy using cyclophosphamide (CPA) is widely associated with antiangiogenesis; however, recent studies implicate other immune-based mechanisms, including antitumor innate immunity, which can induce major tumor regression in implanted brain tumor models. This study demonstrates the critical importance of drug schedule: CPA induced a potent antitumor innate immune response and tumor regression when administered intermittently on a 6-day repeating metronomic schedule but not with the same total exposure to activated CPA administered on an every 3-day schedule or using a daily oral regimen that serves as the basis for many clinical trials of metronomic chemotherapy. Notably, the more frequent metronomic CPA schedules abrogated the antitumor innate immune and therapeutic responses. Further, the innate immune response and antitumor activity both displayed an unusually steep dose-response curve and were not accompanied by antiangiogenesis. The strong recruitment of innate immune cells by the 6-day repeating CPA schedule was not sustained, and tumor regression was abolished, by a moderate (25%) reduction in CPA dose. Moreover, an ~20% increase in CPA dose eliminated the partial tumor regression and weak innate immune cell recruitment seen in a subset of the every 6-day treated tumors. Thus, metronomic drug treatment must be at a sufficiently high dose but also sufficiently well spaced in time to induce strong sustained antitumor immune cell recruitment. Many current clinical metronomic chemotherapeutic protocols employ oral daily low-dose schedules that do not meet these requirements, suggesting that they may benefit from optimization designed to maximize antitumor immune responses.

Neoplasia (2014) 16, 84–96

Introduction

Metronomic chemotherapy involves the administration of cancer chemotherapeutic drugs at regular intervals, without long breaks, and is thought to yield improved antitumor activity through antiangiogenesis combined with conventional drug cytotoxicity [1–4]. Metronomic schedules investigated in preclinical studies include intermittent drug dosing, e.g., the 6-day repeating metronomic schedule empirically found to be most efficacious by Browder et al. [1], as well as daily oral low-dose treatment regimens, which are proposed to be even more effective in killing tumor endothelial cells [5,6]. Metronomic drug schedules have been evaluated in clinical trials, primarily using daily dosing regimens, with promising results [7–9]. Recent studies have shown that other mechanisms, notably antitumor immunity, may also be activated by metronomic chemotherapy. For example, metronomic administration of gemcitabine and docetaxel restores lymphocyte effector function by suppressing bone marrow–derived suppressor cells [10,11], while paclitaxel, cyclophosphamide (CPA), temozolomide and vinorelbine preferentially deplete regulatory T suppressor cells (Tregs) [12–15]. Furthermore, CPA administration

Abbreviations: CPA, cyclophosphamide; MTD, maximum tolerated dose; NK, natural killer; Prf1, perforin-1

Address all correspondence to: Dr David J. Waxman, Department of Biology, Boston University, 5 Cummington Mall, Boston, MA 02215. E-mail: djw@bu.edu

[1]This work was supported in part by grant CA049248 from the National Institutes of Health (to D.J.W.). The authors have no conflicting interest to disclose.

[2]This article refers to supplementary materials, which are designated by Table W1 and Figures W1 to W5 and are available online at www.neoplasia.com.

[3]These authors contributed equally to this work.

Received 20 November 2013; Revised 24 December 2013; Accepted 2 January 2014

DOI 10.1593/neo.131910

on an every 6-day metronomic schedule stimulates antitumor innate immune cell recruitment, which is associated with marked regression of implanted brain tumors in both severe combined immunodeficiency (SCID) immune-deficient and immune-competent mice [16]. Tumor regression is incomplete when natural killer (NK) cells are immunodepleted from fully immune-competent mice bearing subcutaneous syngeneic GL261 gliomas, demonstrating the importance of the innate immune system in tumor regression induced by CPA given on an every 6-day metronomic schedule [16].

Metronomic chemotherapy has shown therapeutic benefits beyond those observed with conventional maximum tolerated dose chemotherapy and can induce responses in patients where standard chemotherapy is no longer effective [7,8]. For example, daily metronomic vinorelbine treatment increases progression-free survival and overall survival in elderly patients with metastatic breast cancer [17]. Intermittent metronomic administration of vinorelbine and cisplatin using optimized doses and schedules gives favorable clinical responses in patients with advanced/metastatic non–small cell lung carcinoma [18], and prolonged responses are achieved in patients with refractory cancers given vinorelbine on a thrice weekly metronomic schedule [13]. A striking 65-month progression-free survival was reported in a patient with stage IIIC ovarian cancer given metronomic CPA on a daily schedule without side effects [19], and metronomic irinotecan administration in patients with metastatic colorectal cancer resulted in stable disease despite progression following initial treatment with a standard irinotecan regimen [20]. Furthermore, metronomic regimens generally show reduced toxicity compared to maximum tolerated dose (MTD) regimens, and by avoiding the dose-dense exposure of MTD schedules, metronomic administration of CPA, as well as other agents, avoids immunosuppression and instead allows for antitumor immune activation [21]. While it is often assumed that the antiangiogenic activity of metronomic chemotherapy contributes to these therapeutic responses, other mechanisms, including antitumor immune responses, may also be at work. Little is known, however, about the requirements, in terms of schedule and dose, to induce metronomic chemotherapy-activated antitumor immunity, a prerequisite for translating preclinical studies of immune-activating metronomic chemotherapeutic regimens to the clinic.

Here, we investigated a recently described brain tumor xenograft model of metronomic CPA-induced antitumor innate immunity [16] to determine the schedule and dose requirements for innate immune cell recruitment and activation leading to tumor regression. We found that tumor recruitment and activation of innate immune cells, including macrophages, dendritic cells, and NK cells, by an intermittent 6-day repeating schedule of metronomic CPA is severely compromised when using dose-equivalent but more frequent metronomic CPA schedules, most notably the daily oral CPA regimen that has served as a model for many clinical trials of metronomic chemotherapy. Furthermore, we report an unexpectedly steep dose-response curve for activation of antitumor innate immunity by the every 6-day metronomic schedule, with a strong correlation seen between innate immune cell recruitment and individual tumor regression responses. Together, our findings support the conclusion that to achieve an effective antitumor immune response, metronomic CPA must be at a dose that is sufficiently high to induce tumor or stromal cell damage leading to the activation of antitumor innate immunity but also sufficiently well spaced in time to not ablate responding antitumor immune cells, some of which may be particularly sensitive to drug cytotoxicity.

Materials and Methods

Cell Lines and Reagents

CPA monohydrate was purchased from Sigma Chemical Co (St Louis, MO). FBS and Dulbecco's modified Eagle's medium (DMEM) culture medium were purchased from Invitrogen (Frederick, MD). The rat 9L gliosarcoma cell line was authenticated by and obtained from the Neurosurgery Tissue Bank at University of California, San Francisco (UCSF; San Francisco, CA) and grown at 37°C in a humidified, 5% CO_2 atmosphere in 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin containing DMEM culture medium.

Quantitative Polymerase Chain Reaction Analysis

Methods for isolation of total RNA from frozen tumor tissue, reverse transcription, and quantitative polymerase chain reaction (qPCR) analysis (primers shown in Table W1 and in [16]) are described [16]. Primers designed using Primer Express software (Applied Biosystems, Carlsbad, CA) were evaluated using LaserGene software (DNAStar, Madison, WI) to ensure mouse gene specificity, except as noted. The absence of cross-species amplification was verified by testing each primer pair on a panel comprising rat liver, human umbilical vein endothelial cells (HUVEC), and mouse liver RNA, in addition to rat 9L and human U251 tumor cell RNA. Data were analyzed using the comparative C_T ($\Delta\Delta C_T$) method and are presented as relative levels of each RNA compared to the RNA level in untreated tumors after normalization to the 18S RNA content of each sample.

Tumor Xenograft Studies

Five- to 6-week-old (24-26 g) male ICR/Fox Chase immune-deficient SCID mice (Taconic Farms, Germantown, NY) were housed in the Boston University Laboratory of Animal Care Facility and treated in accordance with approved protocols and federal guidelines. 9L cells (4×10^6 cells) were injected subcutaneous (s.c.) on each posterior flank in 0.2 ml of serum-free DMEM using a 0.5-inch 29-gauge needle and a 0.3-ml insulin syringe. Tumor areas (length × width) were measured twice weekly using Vernier calipers (VWR, Cat. No. 62379-531), and tumor volumes were calculated on the basis of $\text{vol} = (\pi/6) * (L * W)^{3/2}$. Tumors were monitored, and treatment groups were normalized (each tumor volume was set to 100%) once average tumor volumes reached ~300 mm^3. Mice were treated with CPA given on the following metronomic schedules: oral dosing [23.3 or 70 mg/kg body weight (BW) per day] [6] or intermittent bolus dosing by intraperitoneal (i.p.) injection (70 mg/kg BW, i.p., every 3 days or 70, 105, 140, or 170 mg/kg BW, i.p., every 6 days) on days indicated in each figure by vertical arrows along the *X*-axis. CPA was given orally through drinking water [5] using 0.7 or 2.1 mg of CPA per 4.5 ml of sterile tap water based on a measured water consumption rate of 0.15 ml/g mouse BW per day. All doses are based on the weight of CPA monohydrate. Tumor sizes and mouse body weights were measured at least twice a week. Tumor growth rates before drug treatment were similar among all normalized groups. The plasma pharmacokinetics of 4-OH–CPA exposure was assayed in 9L tumor-bearing mice given CPA by i.p. injection at 70 or 140 mg/kg BW (n = 4 mice per group). We were unable to obtain consistent 4-OH–CPA plasma pharmacokinetic data for the 23.3 mg/kg BW/day oral dosing (drinking water) group [1x per oral (p.o.) dosing], presumably because of the variable time and volume of the last *ad lib* exposure to CPA. To estimate the pharmacokinetics of *ad lib* drinking water exposure to CPA, we assumed that the mice drink five times per day on average. Accordingly, CPA

was administered by oral gavage at 11.65 mg/kg BW (n = 4 mice), corresponding to 20% of the daily doses of 58.25 mg/kg BW (2.5× p.o. dosing). Blood samples were obtained at t = 0 (before CPA exposure) and at t = 5 to t = 240 minutes after CPA dosing by retro-orbital bleeding (100 µl/mouse per time point × four mice), for both the i.p. and the p.o. exposure routes. 4-OH–CPA levels were assayed, and pharmacokinetic parameters were calculated using WinNonlin software [22]. Area under the curve (AUC) values, in units of (nmol/ml) × hour, were normalized to take into account the frequency of administration of each regimen. Thus, "daily AUC" values (Figure 1*C*) were obtained by multiplying the measured AUC values for the 2.5× p.o. group by 5 (cf., 20% of a daily dose given by gavage) and by dividing the measured AUC values of the 70 mg/kg per 3-day i.p. and 140 mg/kg per 6-day i.p. groups by 3 and 6, respectively. The daily AUC of 9.8 (hour × nmol/ml) for 4-OH–CPA exposure measured here in SCID mice given 1× p.o. CPA dosing is very similar to the daily AUC value of 10.3 (hour × nmol/ml) for the same dosing regimen and route (20 mg CPA/kg per day, p.o.) reported by others in C57BL/6J mice [6] (the 56-day AUC value shown in Table 1 of that study was divided by 56 to obtain the daily AUC value).

Tissue Processing and Immunohistochemistry

Tumors were collected on day 6 after the second, third, and fourth cycles of metronomic CPA based on the 6-day repeating schedule,

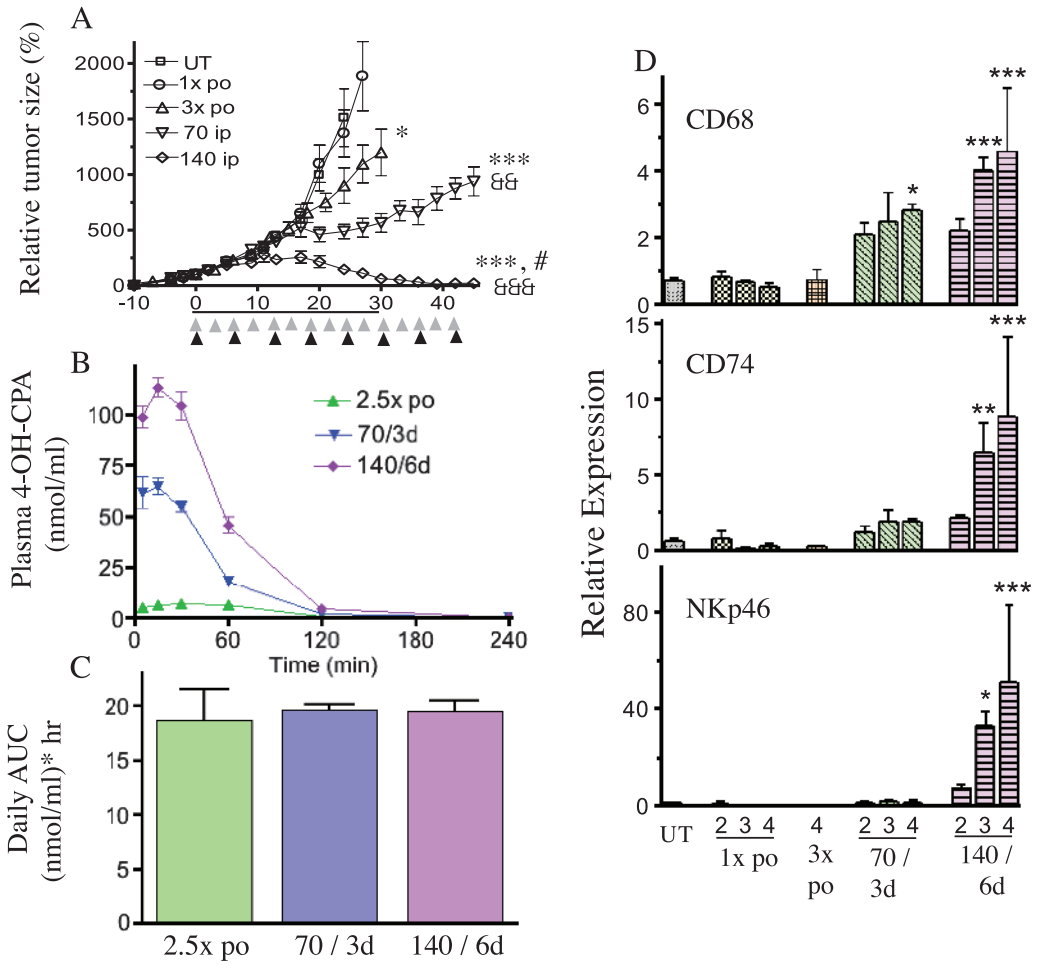

Figure 1. Impact of metronomic schedule on CPA-induced antitumor innate immunity and tumor regression. (A) Growth of 9L xenografts in SCID mice, either untreated (UT) or given metronomic CPA treatment daily, through drinking water at 23.3 mg/kg BW (1× p.o.) or 70 mg/kg BW (3× po) or by i.p. injection every 3 days at 70 mg/kg BW or every 6 days at 140 mg/kg BW. *X*-axis, treatment day. Data are the mean ± SE tumor volumes for n = 5 to 6 mice per treatment group. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared to UT on day 24; $^{\&\&}P < .01$ and $^{\&\&\&}P < .001$ compared to 3× p.o. schedule on day 30; and $^{\#}P < .05$ compared to 70 i.p. schedule on day 30, by one-way ANOVA with Bonferroni multiple comparison correction. (B) Pharmacokinetics of 4-OH–CPA (active metabolite of CPA) levels in plasma of tumor-bearing SCID mice following metronomic CPA treatment using the schedules indicated. (C) Daily AUC for plasma 4-OH–CPA exposure in SCID mice treated with the indicated metronomic CPA regimens, based on mean ± SE values for n = 4 mice per time point, based on the data in B and normalized to adjust for the frequency of administration of each regimen, as described in Materials and Methods section. (D) qPCR analysis of host (mouse) macrophage marker CD68, dendritic cell marker CD74, and NK cell marker NKp46 in 9L tumor xenografts, untreated or treated with the indicated metronomic CPA regimens. Tumors were excised on day 0, and on treatment days 12, 18, and 24 (based on A), corresponding to 6 days after two, three, and four cycles of 6-day repeating CPA treatment, as marked along *X*-axis. Bars, mean ± SE for each treatment group and time point. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, for each CPA treatment schedule and time point (n = 2-5 mice per group) *versus* UT controls (n = 10 mice). Data were analyzed by one-way ANOVA with Bonferroni multiple comparison correction for one tumor randomly selected from each mouse.

i.e., on days 12, 18, and 24 after the first CPA treatment or as indicated in each figure. Tumors were excised and portions were frozen in liquid nitrogen (for RNA, tissue cryosectioning, and immunohistochemistry). A second portion was fixed in 4% paraformaldehyde overnight and then dehydrated in 70% ethanol and embedded in paraffin at the Maine Medical Center Research Institute (Scarborough, ME). Paraffin sections (6 μm) were stained with hematoxylin, proliferating cell nuclear antigen (PCNA), and CD31 after dewaxing with a tissue deparaffinization solution (EZ-DeWax; BioGenex, Fremont, CA). Cryosections to be stained with antibody to CD68, CD31, NK1.1, or perforin-1 (Prf1) were fixed in acetone (NK1.1, Prf1) or 1% paraformaldehyde (CD68, CD31). For both paraffin and frozen sections, endogenous peroxidase was blocked for 5 minutes using 3% hydrogen peroxide and protein blocking was performed for 20 minutes using 2% or 5% normal animal serum from the species where the secondary antibody was raised. Slides were incubated with primary antibody for 1 hour at room temperature (for PCNA and CD31 staining) or overnight at 4°C (for CD68, NK1.1, and Prf1 staining). The following antibodies were used: PCNA (PC10; Cell Signaling Technology, Danvers, MA), anti-mouse CD31 (PECAM-1 antibody, 1:40 dilution for paraffin sections, Cat. No. DIA-310; Dianova, Hamburg, Germany; anti-CD31 antibody, 1:1000 for cryosections, Cat. No. 557355; Pharmingen, San Diego, CA), goat anti-mouse NK1.1 (1:25, CD161, clone M-15, Cat. No. sc-70150; Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse CD68 (MCA1957, 1:100; AbD seroTec, Raleigh, NC), and rat anti-mouse Prf1 (CB5.4, 1:250, Cat. No. sc-58643; Santa Cruz Biotechnology). The following biotinylated secondary antibodies were incubated for 45 to 60 minutes at room temperature at a dilution of 1:200 to 1:250: horse anti-mouse (Cat. No. BA-2000) for PCNA, rabbit anti-goat (Cat. No. BA-5000) for NK1.1, and rabbit anti-rat (Cat. No. BA-4000) for CD31, CD68, and Prf1. An avidin-biotin blocking kit (Cat. No. SP-2001; Vector Laboratories, Burlingame, CA) was used for all incubations, followed by ABC signal amplification with Vector Laboratories Vectastain ABC peroxidase reagent (ABC Elite Kit, Cat. No. PK-6100 or Cat. No. PK-4000). Slides were then submerged in a substrate solution (VIP, Cat. No. SK-4600; ImmPACT VIP, Cat. No. SK-4605; or DAB, Cat. No. SK-4105, all from Vector Laboratories) for 5 to 10 minutes. Hematoxylin solution, Harris Modified (Sigma; Cat. No. HHS128) for 5 minutes, followed by washing with 5% acetic acid for 1 minute and Scott's Tap Water (Sigma; Cat. No. S5134) for 2 minutes, twice, was applied using a BioGenex i6000 Autostainer. Stained tumor sections were visualized under an Olympus BX51 microscope. Tiled images typically covering 4 to 15 nonoverlapping fields were collected (depending on tumor size) and analyzed for at least three randomly selected regions (i.e., ~10-40 images) for each tumor. Images were captured with an Olympus DP25 digital camera using Olympus DP2-BSW software. For stain quantification, images were converted into 8-bit image files, a background threshold was determined using ImageJ software, and all images were processed using an ImageJ software macro to quantify the area of positive stain.

Tumor Excision and Cell Line Preparation

9L tumors were excised from individual mice under sterile conditions in a laminar flow hood and cut to fine pieces (2-4 mm). Fine tumor tissue (100-200 mg) was placed in a gentleMACS tube C containing 8 ml of ice-cold DMEM containing 10% FBS, collagenase type I (450 U/ml; Worthington Biochemical Corp, Lakewood, NJ), and DNase I (0.1 mg/ml, D5025; Sigma) and minced in gentleMACS Dissociator (MACS Miltenyl Biotec, Auburn, CA) using the manufacturer's program "Run m_impTumor_02." The tube was inverted several times and incubated in a 37°C water bath for 20 minutes with shaking. An additional 4500 U of collagenase and 1 mg of DNase I were added, followed by a 20-minute incubation in a shaking 37°C water bath. The sample was reprocessed in a gentleMACS Dissociator using the program "Run m_impTumor_03," centrifuged at 1000 rpm, and filtered through a 70-μm filter into a 50-ml falcon tube. Cells were washed with 10 ml of DMEM and centrifuged twice at 1000 rpm. Cells were resuspended in 1 to 2 ml of DMEM containing 10% FBS, counted, and plated onto two to three plates at different cell concentrations. Colonies comprising ~20 to 200 cells each were counted 9 to 14 days later and collected as passage 0 and then frozen in liquid nitrogen.

Statistical Analysis

Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) with Bonferroni multiple comparison correction, unless indicated otherwise, as implemented in GraphPad Prism 4.1. Fisher exact test was additionally used to compare immune responses in tumors showing complete regression to tumors showing partial regression. Data analysis was carried out using one of the two tumors implanted bilaterally in each mouse unless indicated otherwise. Tumors included in the data presentation and statistical analysis were selected at random (e.g., tumors excised from the right flank of each mouse). Thus, the tumor growth rate data and immune factor expression are based on *n* tumors in *n* mice per treatment group, as specified in each figure legend.

Results

Optimized Intermittent Metronomic Timing Is Essential for Antitumor Immune Activation and Tumor Regression

Metronomic CPA treatment of 9L and several other implanted brain tumor models using an intermittent metronomic schedule (140 mg/kg, i.p., repeated every 6 days) elicits potent antitumor immunity associated with near-complete tumor regression. This regression is achieved in both immunocompetent C57BL/6 mice and in T and B cell–deficient SCID mice [16]. Because innate immune cell activation and the associated tumor regression responses are fully preserved in the adaptive immune cell–deficient SCID mice, which retain the entire suite of innate immune cells, we used this model to further investigate the impact of the metronomic schedule on innate immune cell–based tumor regression. While daily (continuous) low-dose metronomic schedules also show antitumor activity and have most often been used in the clinic in the form of a daily orally administered chemotherapy regimen [7], direct comparisons to the 6-day repeating bolus dose metronomic CPA regimen have not been reported. Daily (oral) administration of CPA at 23.3 mg/kg per day to SCID mice bearing 9L gliosarcoma xenografts did not induce significant tumor growth delay. In contrast, near-complete tumor regression was induced when the same total CPA dose over a 6-day period was given once every 6 days (140 mg/kg, i.p., every 6 days; Figure 1*A*). Pharmacokinetic analysis of plasma drug levels showed, however, that the oral CPA route resulted in underdosing when compared to the every 6-day i.p. regimen and that a 2.5- to 3-fold increase in the nominal oral dose was required to achieve equivalent exposure to the i.p. CPA regimen, as determined by AUC values for exposure to 4-OH–CPA (the active metabolite of CPA; Figure 1*B*) after normalization based on the average total daily drug dose (see Materials

and Methods section; Figure 1*C*). Consequently, we increased the orally administered CPA dose three-fold, to 70 mg/kg per day, to allow for comparison of the daily oral and the every 6-day i.p. schedules at equivalent drug exposures. Nevertheless, only modest tumor growth delay was achieved using the three-fold increased oral CPA dose (Figure 1*A*; 3× p.o. *vs* untreated). Furthermore, only a short period of tumor growth stasis was obtained using an alternative intermittent schedule (70 mg/kg, i.p., every 3 days), followed by tumor regrowth (Figure 1*A*). Thus, the three metronomic CPA treatment schedules give an unexpectedly wide range of antitumor responses, despite their equivalence with respect to net exposure to active drug.

Analysis of tumor tissue showed that the daily CPA regimen failed to induce tumor recruitment of macrophages, dendritic cells, or NK cells, as revealed by qPCR analysis of their respective markers (CD68, CD74, and NKp46; 1× p.o. and 3× p.o.; Figure 1*D*). The 70 mg/kg every 3-day regimen showed increased recruitment of macrophages but not dendritic cells or NK cells, whereas the 140 mg/kg every 6-day schedule activated broad-spectrum antitumor innate immunity, characterized by significant recruitment of all three classes of immune cells (Figure 1*D*). These findings were verified by immunohistochemical analysis of infiltrating immune cell populations in cryosections prepared from tumors excised on treatment day 24 (Figures 2*A* and W1). Recruitment of macrophages (CD68 staining) was evident in the every 3-day and every 6-day CPA-treated tumors, while NK cells (NK1.1 and perforin staining) were evident in the every 6-day treated tumors only. Quantitation of these results showed that only the 140 mg/kg every 6-day schedule significantly increased tumor-associated macrophages and NK cells (Figure 2*B*). Overall, the antitumor response of each metronomic CPA schedule correlated with the extent of recruitment of one or more of immune cell classes (Figure 1*A* *vs* Figure 1*D*). Thus, the activation of innate immune cell recruitment by metronomic CPA that we reported previously [16] requires CPA administration on an intermittent metronomic schedule, with sufficient time (i.e., 6 days) between cycles of drug administration.

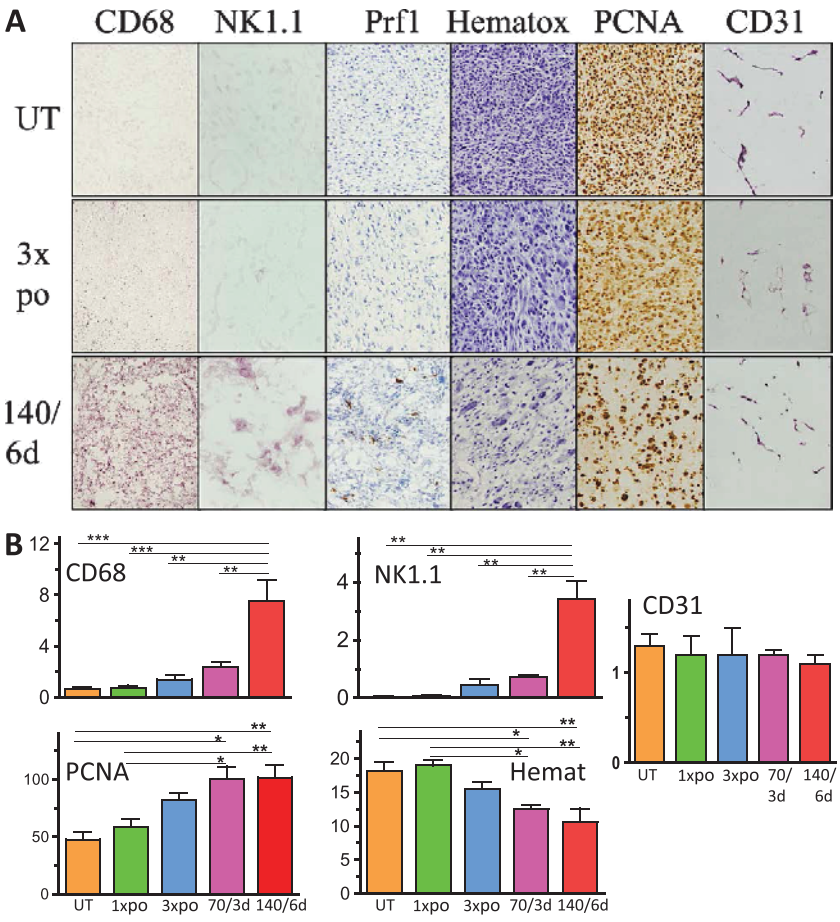

Figure 2. Metronomic CPA regimens have differential effects on antitumor innate immunity: (A) 9L tumor cryosections immunostained for macrophage marker CD68 (×4.2), NK cell marker NK1.1 (×40), and NK cell effector perforin (Prf1; ×20). Tumor paraffin sections were stained with hematoxylin for cell density, PCNA (cell proliferation), and endothelial cell marker CD31 (all at ×20). Tumors (excised day 24): untreated or CPA treated [140 mg/kg BW, every 6 days and through drinking water (3× p.o.); as in Figure 1]. Representative photos based on three to four independent tumor sections from each of $n = 3$ tumors randomly selected from $n = 3$ mice per group for CD68, NK1.1, and Prf1 and $n = 2$ to 5 mice per group for the histochemical stainings are shown. See also Figure W1. (B) ImageJ quantification of immunostained macrophage marker CD68, NK cell markers NK1.1 and Prf1, and endothelial cell marker CD31, hematoxylin staining, and PCNA staining normalized by relative cell density (i.e., hematoxylin staining) in untreated (UT) 9L tumors and in metronomic CPA-treated tumors using the schedules indicated; tumors were analyzed on CPA treatment day 24. Error bars, mean ± SE for $n = 3$ to 6 tumors per group, based on data averaged over 10 to 40 images analyzed per tumor. *$P < .05$, **$P < .01$, ***$P < .001$ compared to UT or CPA treatment, as indicated, analyzed by one-way ANOVA with Bonferroni multiple comparison correction.

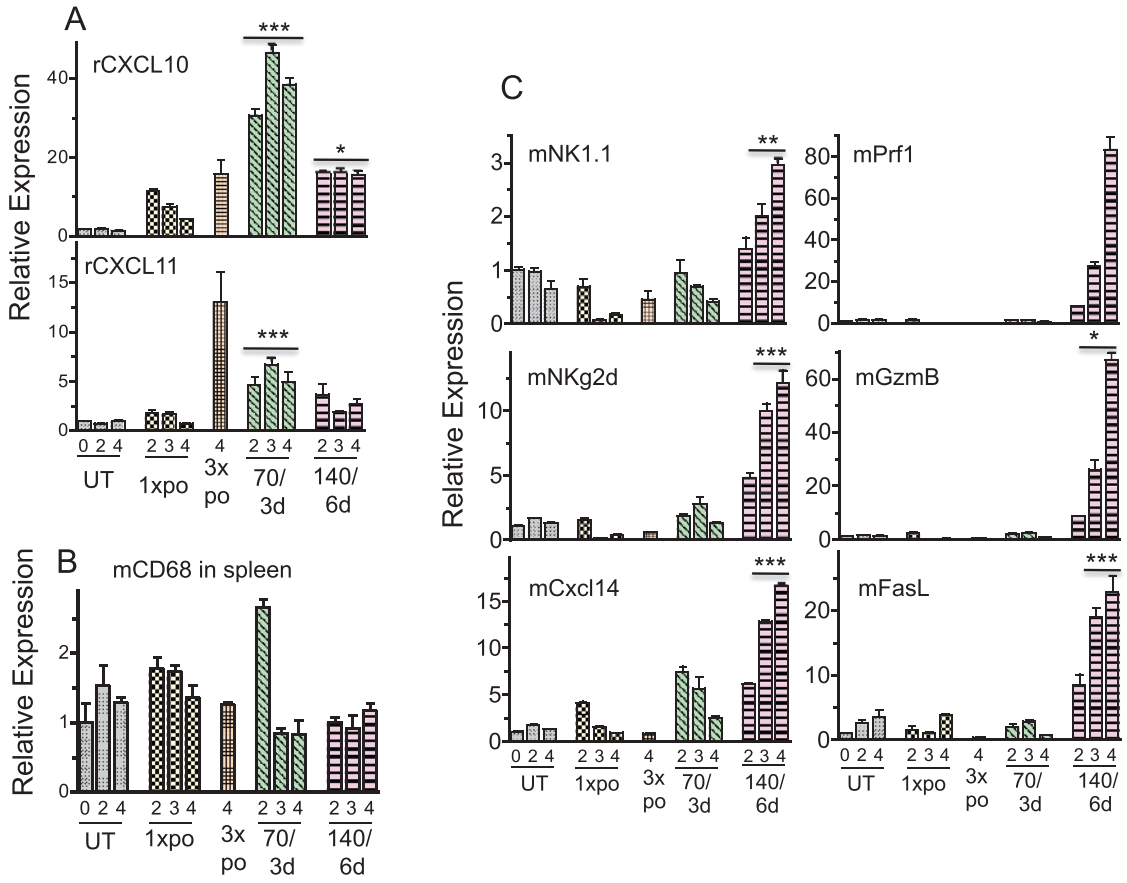

Figure 3. Metronomic CPA regimens have differential effects on chemokines, cytotoxic effectors, and immune markers. (A) Tumor cell expression of chemokines CXCL10 and CXCL11 in 9L tumor xenografts treated as in Figure 1 and analyzed using rat (r)–specific qPCR primers on pooled tumor RNA samples collected on treatment days 12, 18, and 24 (6 days after two, three, and four cycles of 6-day repeating CPA treatment, as marked). (B) Expression of CD68 (macrophage marker) in spleens of tumor-bearing mice assayed in A. (C) Expression of additional mouse immune markers in tumors analyzed as in A: NK cell marker NK1.1, NK cell effectors perforin (Prf1) and GzmB, activating receptor NKG2D, CXCL14, and Fas ligand (FasL). Error bars, mean ± SD for $n = 3$ replicate assays of samples collected from tumors or spleens from $n = 2$ to 4 mice per treatment group. *$P < .05$, **$P < .01$, and ***$P < .001$ for comparisons of each CPA schedule after two to four treatment cycles (days 12-24; A) or after three to four treatment cycles (days 18-24; C), compared to UT tumor controls, by one-way ANOVA with Bonferroni multiple comparison correction. In A, both the 70/3-day and the 140/6-day CPA schedules were significantly different from untreated controls, whereas in C, only the 140/6-day schedule showed significant differences, as marked.

Differential Effects of Metronomic Schedules on Tumor Microenvironment and Innate Immune Cell Recruitment

To better understand the differential antitumor effects of each metronomic CPA schedule, we investigated the expression of factors that may be important for the observed antitumor responses. First, we assayed the ability of each drug schedule to induce tumor cell damage leading to initiation of a stress-mediated antitumor immune response, as judged by tumor (rat) cell–specific expression of the inflammatory chemokines CXCL10 and CXCL11, which are activated in response to damage associated with cytotoxic tumor cell stress [23] and can promote chemotaxis of activated NK cells [24]. Tumor cell expression of CXCL10 was significantly increased by both the every 3-day and the every 6-day CPA treatments, with similar increases (albeit not statistically significant) seen with the daily CPA regimen. Similar patterns were seen for CXCL11 (Figure 3*A*). Accordingly, the ability of CPA to stimulate cytotoxic tumor cell stress, as measured by these markers, does not account for the CPA schedule dependence of immune cell recruitment and antitumor responses. Next, we investigated whether the three metronomic schedules have differential cytotoxic effects on splenic immune cell reservoirs. Macrophage levels were not affected by any of the metronomic regimens (Figure 3*B*), suggesting that splenic macrophages are insensitive to CPA, in agreement with our earlier finding with the every 6-day metronomic schedule [16]. However, splenic levels of dendritic cell and NK markers were generally decreased by all three CPA treatment regimens (Figure W2). Interestingly, the neutrophil marker Gr1 showed the greatest decreases with the every 3-day metronomic schedule, indicating a schedule-dependent sensitivity to CPA (Figure W2).

The dependence of the NK cell response on the schedule of metronomic CPA treatment was confirmed by analyzing the NK cell marker NK1.1 and the NK cell receptor NKG2D, as well as the NK cell effectors perforin (Prf1) and granzyme B (GzmB), which consistently showed the greatest responses to the every 6-day metronomic CPA schedule (≥60-fold increases after four cycles; Figure 3*C*). Moreover, in contrast to the responses to the tumor cell damage/stress response chemokines CXCL10 and CXCL11, which were already maximal after the second cycle of metronomic CPA (i.e., day 12 after the first CPA treatment), the NK cell markers and cytotoxic effectors showed progressive increases with each additional every 6-day CPA injection. The host cell–expressed chemokine CXCL14, which is a chemoattractant

for macrophages, immature dendritic cells, and NK cells [25], was significantly induced by the 6-day metronomic schedule only (Figure 3*C*). Finally, Fas ligand, which potentiates cell death by activating Fas receptor, showed a pattern of response similar to that of perforin and GzmB, namely, only the 6-day metronomic regimen showed significant activation. Fas ligand increases macrophage cytotoxicity [26] as well as NK cell contact-mediated cell death [27]. *In vitro* analyses confirmed that cultured 9L tumor cells are intrinsically sensitive to NK cell cytotoxicity, as shown using NK92 cells activated with interleukin 2 (Marie Jordan, unpublished experiments).

Next, we investigated whether the three metronomic schedules induced other differential effects within the tumor. Tumor cell density, assayed by hematoxylin staining 24 days after the first CPA treatment, was significantly decreased by both the 3-day and the 6-day metronomic CPA schedules but not by the continuous daily regimens (Figures 2, *A* and *B*, and W1*B*). Unexpectedly, significant increases, rather than decreases, in cell proliferation were seen with the 6-day metronomic CPA schedule, as indicated by PCNA immunostaining normalized to the hematoxylin-staining intensity (Figure 2*B*). Intense PCNA staining was seen in both the every 3-day and every 6-day CPA-treated tumors, but overall, staining was restricted to a much smaller fraction of the tumor section area than in the daily CPA-treated tumors and the untreated tumor controls (Figures 2*A* and W1*B*). This increase in PCNA staining, as well as the change in cell morphology apparent in the hematoxylin and PCNA-stained tissue sections, may be indicative of the tumor-infiltrating immune cells [28,29]. Finally, none of the metronomic CPA regimens caused a significant decrease in tumor microvessel density, as evidenced by CD31 staining (Figures 2 and W1*B*), indicating that metronomic chemotherapy does not act through an antiangiogenic mechanism in the case of these established and well-vascularized tumors. Therefore, the observed regression of the 6-day metronomic CPA-treated tumors, and the loss of tumor cellularity, cannot be explained by metronomic chemotherapy-induced antiangiogenesis.

Metronomic CPA Dose Optimization for Sustained Antitumor Innate Immune Cell Recruitment

Next, we investigated the dose dependence of the antitumor responses to the 6-day metronomic CPA treatment schedule (Figure 4*A*). Antitumor activity was characterized by an unusually steep dose dependence, with modest tumor growth delay seen at every 6-day doses of 70 mg CPA/kg, initial tumor stasis followed by tumor regrowth at 105 mg CPA/kg, and major tumor regression after a delay of about two to three treatment cycles at 140 and 170 mg CPA/kg. Investigation of infiltrating immune cell markers revealed no significant immune cell recruitment at 70 mg/kg CPA. Several immune markers were significantly increased after four cycles of metronomic CPA (treatment day 24) at the 105 mg/kg dose, at which time the tumors were nearly growth static; however, these immune responses declined with further cycles of CPA treatment (Figure 4*B*, seven CPA cycles) in association with the resumption of tumor growth (Figure 4*A*). In contrast, the innate immune cell responses were sustained through seven CPA cycles at both the 140 and 170 mg/kg doses (Figure 4*B*) in association with the observed patterns of marked tumor regression (Figure 4*A*). These

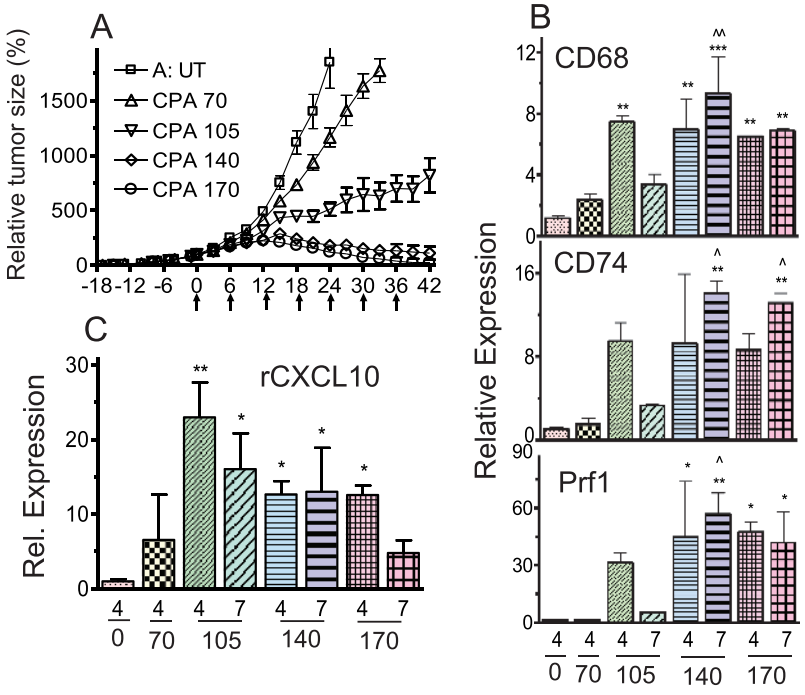

Figure 4. Impact of dose of metronomic/6-day CPA schedule on tumor response and innate immune recruitment. (A) Tumor volumes for 9L xenografts, either untreated (UT) or given metronomic CPA treatment every 6 days (black arrows) at 70, 105, 140, or 170 mg CPA/kg BW per i.p. injection. Error bars, mean ± SE for $n = 10$ to 12 mice per treatment group. (B, C) Expression of the indicated immune cell and chemokine markers in tumors assayed on day 6 after either four or seven CPA cycles, corresponding to treatment days 24 and 42 in A. Error bars, mean ± SE for $n = 2$ to 4 mice per treatment group. *, **, ***$P < .05$, .01, and .001, respectively, *versus* UT (CPA dose of 0 mg/kg BW). ^, ^^$P < .05$ and .01, respectively, *versus* CPA dose of 105 mg/kg BW treatment on day 6 after seven cycles of CPA treatment. Data were analyzed by one-way ANOVA with Bonferroni multiple comparison correction for one tumor randomly selected from each mouse (B and C). See also Figure W3.

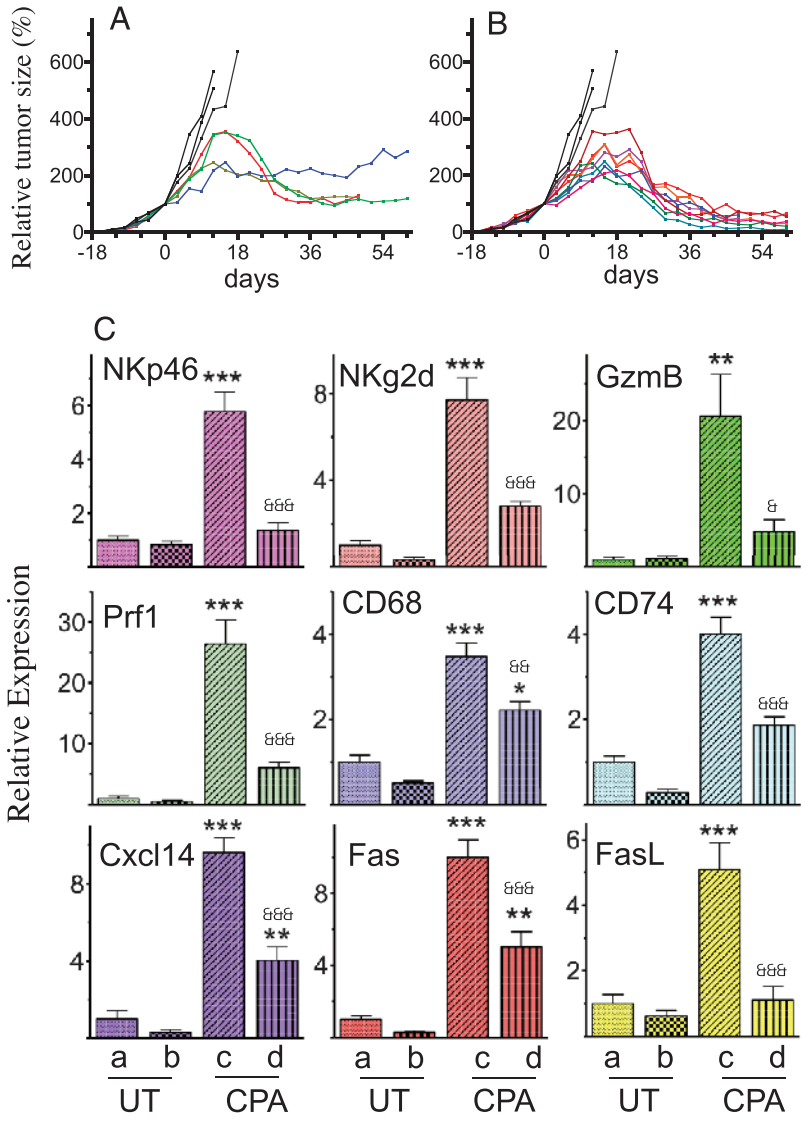

Figure 5. Relationship between innate immune recruitment and regression of 9L tumor xenografts. (A, B) Volumes of individual 9L tumors that were untreated (UT; $n = 3$ tumors from three mice) or were given metronomic CPA at 140 mg/kg ($n = 12$ tumors). Four tumors that partially regressed or were growth static are shown in A, and eight fully or near-fully regressing tumors are shown in B. (C) qPCR analysis of tumor expression levels of the indicated factors. Data shown are for untreated 9L tumors on treatment day 0 (a, $n = 6$) or treatment day 24 (b, $n = 6$) and on day 6 after either 8 or 10 cycles of CPA treatment for fully responsive tumors (as in B; c, $n = 8$) and for the partially regressing tumors (as in A; d, $n = 4$). *, **, ***$P < .05$, .01, and .001, respectively, *versus* UT on day 0 (a). &, &&, &&&$P < .05$, .01, and .001, respectively, for partially regressing tumors (d) *versus* more completely regressing tumors (c), analyzed by one-way ANOVA (nonparametric) with Bonferroni multiple comparison correction. The partially regressing tumors (d) were also significantly different from the fully regressing tumors (c) when evaluated using Fisher exact test: CD68 and CD74, $P < .02$; FasL, $P < .005$; NKp46, NKG2D, Cxcl14 and Fas, $P < .001$; GzmB and Prf1, $P < .0001$. See also Figure W5.

findings were verified using a more complete panel of NK cell markers as well as Fas ligand (Figure W3).

Significant increases in tumor cell expression of CXCL10 were seen at metronomic CPA doses of 105, 140, and 170 mg/kg. Maximum increases in CXCL10 occurred at the 105 mg/kg dose (Figure 4*C*), perhaps reflecting the decreases in tumor cell density at the higher CPA doses (Figure W4). This finding is consistent with our conclusion, based on Figure 3*A*, that CXCL10 induction marks tumor cell exposure to activated CPA but is not predictive of immune cell recruitment or antitumor response. Furthermore, significant and sustained increases in the NK cell–recruiting host (mouse) chemokine CXCL14 were seen at the metronomic CPA doses of 140 and 170 mg/kg, with the 105 mg/kg dose inducing an initial but transient elevation of CXCL14 (Figure W3).

Robust Innate Immune Recruitment Is Required for Complete Tumor Regression

During the course of these studies, we noted several individual 9L tumors that initially regressed when treated with CPA at 140 mg/kg on the every 6-day schedule but that did not regress further (i.e., tumors became growth static), despite ongoing metronomic CPA treatment (Figure 5*A*, partially regressing tumors *vs* Figure 5*B*, fully regressing tumors). Other individual tumors exhibited growth delay without regression (Figure 5*A*), similar to tumors treated with CPA

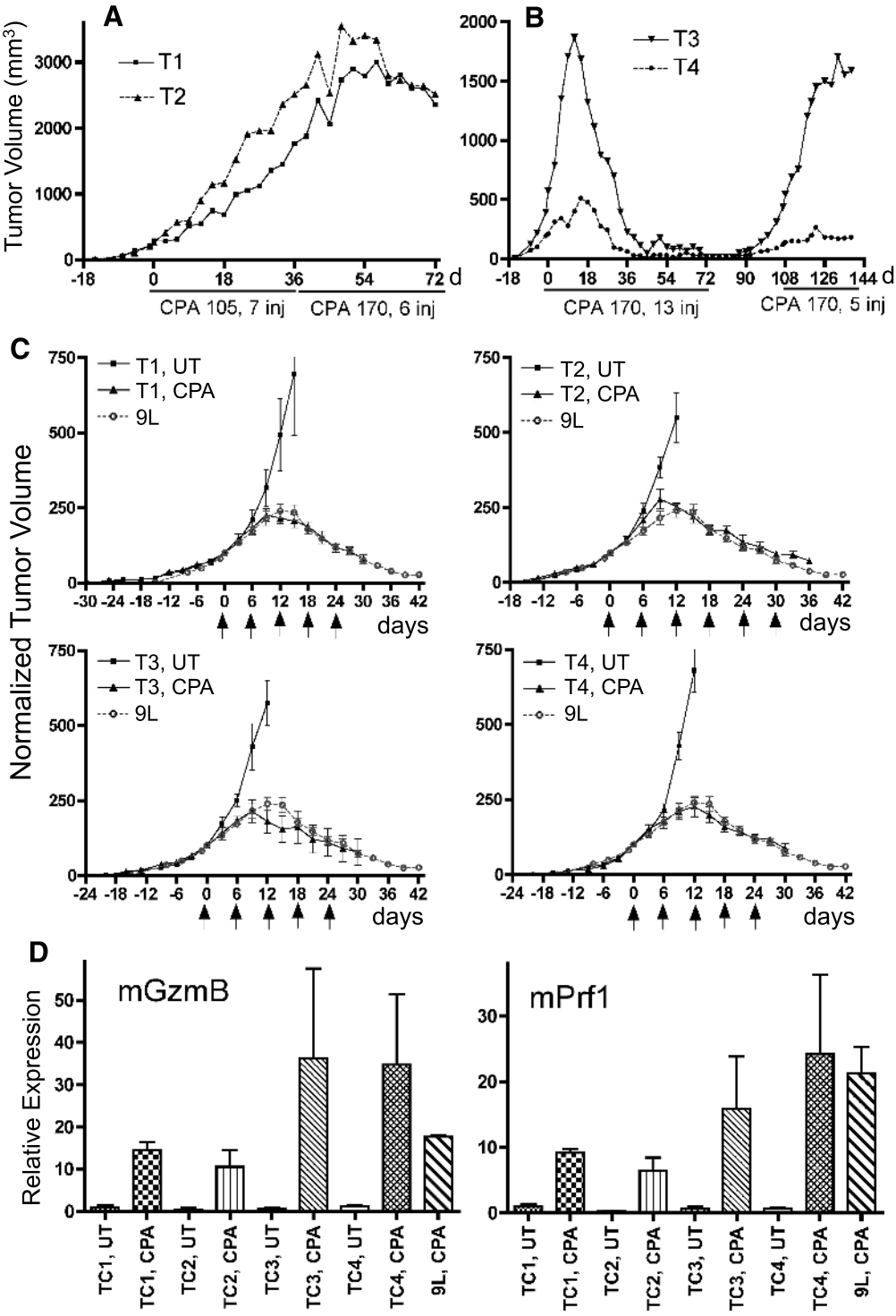

Figure 6. Analysis of tumors unresponsive to second round of metronomic CPA treatment. (A) Tumor growth curves for two individual 9L tumors treated with CPA at 105 mg/kg BW per i.p. injection for seven CPA cycles, which induced tumor growth delay (cf. Figure 4*A*), followed by six additional metronomic CPA cycles at 170 mg/kg BW per i.p. injection, which induced tumor growth stasis but not major regression. (B) Tumor growth curves for two individual 9L tumors treated with CPA at 170 mg/kg BW per i.p. injection for 13 CPA cycles, which induced tumor regression (as in Figure 4*A*), followed by a break of 36 days. CPA treatment was reinitiated on day 108 and continued for five additional cycles at 170 mg/kg BW per i.p. injection, which induced tumor growth stasis but not major regression. (C) 9L tumor sublines derived from individual tumors T1, T2, T3, and T4 (as in A and B) were grown in SCID mice. The responsiveness of each tumor subline to CPA treatment at 170 mg/kg BW per i.p. injection beginning on treatment day 0, when mean tumor volumes were 350 to 400 mm^3, is shown. Tumor growth data are based on one randomly selected tumor from each of $n = 4$ to 5 mice per treatment group. Tumor volumes shown are mean ± SE values, normalized to 100% on day 0. 9L, growth curve for parental 9L tumors, shown as a reference. (D) qPCR analysis of expression levels of host (m, mouse) NK cell effectors GzmB and perforin (Prf1), on day 6 after either five CPA treatment cycles (T1, T3, and T4 tumors) or six CPA cycles (T2 tumors). Error bars, mean ± SD for $n = 3$ replicate assays of samples from tumors collected from $n = 4$ to 5 mice per treatment group.

at 105 mg/kg every 6 days (Figure 4*A*). Characterization of the incompletely responsive 9L tumors revealed a level of innate immune cell recruitment significantly lower than in the more completely regressing tumors from the same study, as indicated by reduced levels of several NK cell markers (NKp46, NKG2D, GzmB, Prf1; Figure 5*C*, tumor groups d *vs* c). More modest reductions in infiltrating macrophage (CD68) and dendritic cells (CD74), in the chemokine CXCL14, and in Fas and FasL, were also seen in the incompletely responsive tumors compared to the fully regressing tumors. An inverse relationship between tumor volume and the expression level of these innate immune-related markers was seen across the full set of 12 individual tumors (Figure W5), further highlighting the relationship between immune cell recruitment and tumor regression.

Next, we investigated whether tumor regression could be achieved in the 105 mg/kg every 6-day CPA treatment group (Figure 4*A*) by increasing the dose of CPA after seven CPA cycles. Figure 6*A* shows that tumor growth stasis, and not tumor regression, was achieved when CPA was administered at a higher dose, 170 mg/kg per 6 days, for six additional cycles (days 36-72). In other experiments, several tumors that showed strong and apparently complete regression following CPA treatment at 170 mg/kg every 6 days began to regrow when CPA treatment was discontinued for a period of 36 days (Figure 6*B*). A second round of tumor regression was not achieved, however, when metronomic CPA treatment was reinitiated at the same dose and schedule (170 mg/kg every 6 days; Figure 6*B*, days 108-132). This could reflect depletion of immune cell reservoirs (cf. Figure W2), or alternatively, it may involve immunosuppression, or acquired tumor cell resistance to CPA.

To investigate the possibility that acquired tumor cell resistance is responsible for the observed tumor insensitivity to a second round of metronomic CPA treatment, tumors were excised and tumor cell lines were prepared from the four individual tumors shown in Figure 6, *A* and *B* (tumors T1-T4). All four tumor cell lines derived from the *in vivo* metronomic CPA-resistant tumors showed a sensitivity to activated CPA (EC_{50} = 5.9-8.3 μM) only moderately lower than that of parental 9L tumor cells [half maximal effective concentration (EC_{50}) = 3.7-4.1 μM] when assayed in a 4-day cell culture growth inhibition assay using chemically activated CPA (4-hydroperoxy–CPA). Thus, prolonged metronomic CPA treatment *in vivo* does not induce strong intrinsic tumor cell resistance to activated CPA. Next, we investigated the responsiveness of the four tumor sublines to metronomic CPA-induced immune cell recruitment and tumor regression after reimplantation in tumor-naïve mice. All four 9L sublines yielded tumors that showed consistent regression following metronomic CPA treatment *in vivo* (170 mg/kg per 6 days; Figure 6*C*). Moreover, in each case, a strong antitumor innate immune response was observed, marked by strong increases in the cytotoxic NK cell effectors GzmB and perforin (Figure 6*D*).

Discussion

Metronomic CPA delivered using an every 6-day schedule activates a potent antitumor innate immune response that leads to major regression of established brain tumor xenografts [16]. Here, we show that this immune response is not recapitulated when using a more frequent schedule of metronomic CPA administration, in particular, a daily oral regimen [5], that results in the same net drug exposure and that has served as a model for many clinical trials of metronomic chemotherapy [7,9]. While the antiangiogenic actions of metronomic CPA, and other agents, may contribute to antitumor activity in certain tumor models [1,3], no antiangiogenesis was observed using either the intermittent or the daily metronomic schedules investigated in this study. As such, metronomic chemotherapy-induced antiangiogenic effects are likely drug and tumor model dependent. These findings support our conclusion that innate immune recruitment, and not antiangiogenesis, is the major mechanism for the striking tumor regression that is induced by metronomic CPA when given on a 6-day repeating schedule in these subcutaneous brain tumors, as seen in both SCID mice and in fully immune-competent mice [16].

The empirical observation that a 6-day metronomic CPA schedule is optimal with regard to antitumor activity [1] could reflect the life-span of host immune cells or inflammation initiators such as platelets, which are first-line immune responders to tissue damage and have a life-span of 5 to 10 days [30]. While other metronomic chemotherapy schedules, including daily low-dose regimens, show improved antitumor activity in some models [7], our findings indicate that the every 6-day metronomic schedule used here may be optimal with respect to activation of innate immunity: sufficiently frequent to repeatedly induce the tumor cell cytotoxicity and inflammation needed to activate cytokine/chemokine attractants that stimulate an innate immune response and sufficiently well spaced in time so as to minimize the killing of chemosensitive immune cells recruited to the tumor. Thus, intermittent (every 6 days) delivery of CPA as a bolus injection may help ensure that a sufficient level of active drug is present within the tumor microenvironment to induce cellular damage and stimulate the production of immune cell chemoattractants. Further supporting a minimum dose and tumor damage threshold requirement, mathematical modeling comparing both conventional and metronomic administration of temozolomide showed how lowering drug dosage could lead to a decrease in therapeutic effect [31].

The striking difference in efficacy between daily low-dose and intermittent bolus-dose metronomic CPA (Figure 1*A*) is not due to differences in the route of administration (p.o. *vs* s.c.) or a dose dependence of liver P450 enzyme–catalyzed prodrug activation (CPA 4-hydroxylation) [32], insofar as these comparisons between metronomic schedules were carried out at the same net level of drug exposure, as indicated by daily plasma AUC values (Figure 1*C*). The AUC level of active circulating drug generated here using low-dose (daily p.o.) metronomic CPA was comparable to that reported previously for the same metronomic schedule and route [6], where significant tumor growth delay (albeit no tumor regression) was achieved in an s.c. prostate cancer PC-3 xenograft model. It should be noted, however, that the significant tumor growth delay activity of daily metronomic CPA seen in those studies and in several other tumor models [5,6] was achieved when CPA treatment was initiated when the tumors were small (up to ~100 mm^3) and perhaps particularly sensitive to antiangiogenesis-induced growth delay. In contrast, in the present study daily metronomic CPA treatment was initiated when the tumors were well established (~300 mm^3) and already well vascularized and perhaps no longer hypersensitive to metronomic CPA-induced antiangiogenesis. The glioma models used here (9L) and in our earlier work (9L, U251) [16] are not resistant to antiangiogenesis *per se*; indeed, they are highly sensitive to antiangiogenesis induced by targeted inhibitors of vascular endothelial growth factor receptor [16,33]. These findings indicate that daily metronomic CPA is comparatively weak as an antiangiogenic agent, at least when assayed using these highly vascular s.c. glioma models.

The schedule dependence of metronomic chemotherapy-induced immune cell activation is likely to vary and will need to be optimized for each drug. In addition to metronomic timing, optimized drug dosage was found to be crucial for eliciting effective antitumor immunity and tumor regression. Thus, the strong recruitment of innate immune cells stimulated by metronomic CPA at 140 mg/kg per 6 days was not sustained, and tumor regression was abolished, by as little as a 25% reduction in CPA dose, with clear differences in effects on tumor cellularity and proliferative capacity seen at higher (140 mg/kg) compared to lower (70 and 105 mg/kg) CPA doses. Increasing the CPA dose by ~20%, to 170 mg/kg per 6 days, eliminated the partial tumor regression and weak innate immune cell recruitment seen in a subset of the every 6-day treated tumors. These steep dose dependencies suggest a need for a critical threshold of DNA damage or cellular stress to initiate antitumor immunity and tumor regression. The dose dependence and kinetics of onset of cell damage that triggers antitumor immunity is likely to vary between tumors, as indicated by our finding that 9L gliosarcomas treated with CPA at 140 mg/kg per 6 days do not begin to regress until the third treatment cycle (Figure 1*A*) [33,34], whereas tumor regression begins shortly after the first CPA cycle in the U251 glioblastoma and GL261 glioma models [16].

9L tumors that did not regress at a CPA dose of 105 mg/kg per 6 days failed to regress when subsequently treated with CPA at a dose (170 mg/kg per 6 days) that readily regressed CPA-naïve tumors. Moreover, when tumors that regressed following every 6-day CPA treatment were allowed to regrow, they did not regress on reinitiation of the same treatment regimen. Tumor cells derived from these "metronomic CPA-resistant" tumors (Figure 6) were, however, intrinsically sensitive to activated CPA when assayed in cell culture, and they exhibited normal metronomic CPA-induced regression patterns when reimplanted in tumor-naïve mice. The loss of tumor responsiveness could reflect the emergence of an immunosuppressive tumor environment, or may be due to an impaired host innate immune response after many cycles of CPA treatment, consistent with the observed depletion of splenic immune cell reservoirs (Figure W2). This loss of responsiveness is not due to the recruitment of Tregs, which are absent in SCID mice. Furthermore, even in C57BL/6 mice, where Tregs are expressed, the Treg population was not increased after several cycles of metronomic CPA treatment at 140 mg/kg per 6 days [16], consistent with other reports that metronomic chemotherapy depletes and/or inhibits Tregs [12–15]. Another possible cause for the apparent loss in responsiveness in our SCID mice could be induction of $Gr1^+CD11b^+$ bone marrow or myeloid-derived suppressor cells, which can suppress antitumor immunity. However, we have found that metronomic CPA treatment (140 mg/kg per 6 days) decreases, rather than increases, $Gr1^+CD11b^+$ cell numbers in both spleen and bone marrow and does not significantly increase these cells in treated 9L tumors (our unpublished experiments). Taken together, these findings suggest that depletion of sensitive innate immune cell populations and/or development of an immunosuppressive tumor microenvironment is the cause for loss of efficacy following long-term metronomic CPA treatment. Further studies will be required to ascertain whether a similar loss of responsiveness occurs in fully immune-competent animal models and in immune-competent patients.

Several mechanisms may contribute to metronomic CPA-induced innate immune cell recruitment leading to tumor regression. Apoptosis, which is induced by CPA [35], can be immunogenic [36]. Further, one or more families of NK cell ligand-receptor pairs [37] may mediate NK cell recruitment. This could involve an increase in expression of NKG2D ligands following cytotoxic damage [38], as indicated by the increase in the NKG2D ligand MICB in U251 glioblastoma treated with metronomic CPA every 6 days [16]. Chemotherapeutic drugs could also activate antitumor immunity through their effects on transcriptional regulators of immune response factors. For example, 5-fluouracil increases thrombospondin-1, implicated in tumor macrophage recruitment [39], by up-regulation of Egr1, an activator of the thrombospondin-1 promoter [40]. CPA can also elicit a macrophage-associated antitumor response by activating a DNA damage response pathway leading to tumor-specific secretion of high mobility group box 1 protein [41]. Metronomic CPA-induced persistent DNA damage may activate the senescence-associated secretory phenotype, with secretion of factors that stimulate antitumor innate immunity [42]. Further, we observed metronomic CPA activation of tumor cell–expressed CXCL10 and CXCL11, which may contribute to immune cell infiltration. CXCL10 is activated by cytotoxic tumor cell stress [23] and both CXCL10 and CXCL11 can promote chemotaxis of activated NK and T cells [24]. However, NK cell recruitment did not occur when these factors were induced by the daily and 70 mg/kg per 3-day metronomic CPA regimens (Figure 3*A*), indicating these factors alone are not sufficient to activate the antitumor innate immune response. Conceivably, the intrinsic capacity of CXCL10 and CXCL11 to induce NK cell recruitment [24] may be countered by the killing off of the responding NK cells due to their frequent exposure to activated CPA when using the daily and the every 3-day CPA schedule.

While daily low-dose metronomic chemotherapy regimens may be convenient and are widely employed in the clinic [7,9], they may not always be optimal with regard to therapeutic effectiveness. Our studies here show that the intermittent (every 6 days) bolus-dose approach to metronomic chemotherapy, originally introduced by the Folkman Laboratory [1], is mechanistically distinct, insofar as it provides for drug-free breaks, which spare drug-sensitive innate immune cells essential for a robust antitumor immune response. Other intermittent metronomic drug schedules that show significant antitumor activity include intermittent treatment with paclitaxel and epothilone drugs (every 6, 8, 9, 12, and 14 days), which fully regress mammary, neuroblastoma, and ovarian tumor xenografts grown in nude mice [43]. Furthermore, intermittent metronomic chemotherapy can be safely administered for prolonged periods of time [13]. While preclinical models show that daily low-dose metronomic chemotherapy is also well tolerated, tumor growth delay or, in some cases, tumor stasis is a common outcome and major sustained tumor regression is rarely achieved, in contrast to our findings with intermittent metronomic dosing of CPA. For example, daily oral low-dose metronomic CPA induces only modest tumor growth delay in breast 231/LM2-4 tumors grown orthotopically in nude mice [44]. Daily low-dose CPA induces only an ~20-day growth delay of orthotopic 231-H2N mammary tumors [45], while PC-3 prostate cancer xenografts initially regress partially, then become static, and then begin to regrow about 25 days later [5]. As the latter treatment was initiated when the PC-3 tumors were relatively small, the partial regression observed could reflect an initial antiangiogenic response. In that same study, daily CPA treatment induced a very modest growth delay of MDA-MB-231 xenografts grown orthotopically in SCID mice [5].

The present findings have major implications for the design of metronomic chemotherapeutic regimens in the clinic, where there is a need for biomarkers to assess and optimize dose and schedule for effective activation of immune responses. Many cancer chemotherapeutics have the potential to stimulate immune responses, even in the absence

of metronomic scheduling. Thus, CPA promotes increased bone marrow generation of dendritic cell precursors capable of antigen presentation [46] and differentiation of T helper 17 cells in both rodents and humans [47], vinblastine induces dendritic cell maturation [48], and the therapeutic efficacy of doxorubicin depends on CD8 T-cell activation and interferon-γ production [49]. Antitumor immune activation may thus be an important mechanism of action for many anticancer agents, including other conventional chemotherapeutics that show efficacy when administered in metronomic schedules [48,50], and warrants further study for its potential to elicit strong innate antitumor immune responses in the clinic. Given the increased clinical use of daily metronomic schedules of CPA and other anticancer drugs, our finding that daily metronomic dosing of CPA does not recapitulate the major innate immune recruitment that can be achieved using an intermittent metronomic schedule should be taken into consideration when developing immune-activating metronomic drug schedules for evaluation in the clinic.

Acknowledgments

We thank Jeannette Connerney for assistance in collecting blood samples by retro-orbital bleeding.

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Table W1. Oligonucleotide Primer Sets Used for qPCR Analysis.

Gene	qPCR Primers (5′ to 3′)
Chemokine CXCL10	Forward: 5′-GAATCTGAGGCCATCAAGAGCTTAT-3′
(*Cxcl10*) (rat-specific)	Reverse: 5′-TGTCCATCGGTCTCAGCACTGT-3′
Chemokine CXCL11	Forward: 5′-GCTATGATCATCTGGGCCACAA-3′
(*Cxcl11*) (rat-specific)	Reverse: 5′-GGGTAAATTACAGAAACTTCCTTGATTG-3′
Fas ligand (*Fasl*, *Cd95l*)	Forward: 5′-CTGGCAGAACTCCGTGAGTTC-3′
(mouse-specific)	Reverse: 5′-CCACACTCCTCGGCTCTTTTT-3′

Primers were designed to anneal at their 3′ end in a mouse (host)–specific manner, except as noted. Species alignments between human, rat, and mouse sequences were used for each gene to determine primer set specificity. The absence of cross-species amplification was verified by testing primer sets on a panel of rat, mouse, and human RNA to ensure species specificity. Gene names are shown in parentheses. Sequences for the following mouse-specific primer sets were reported previously [16]. Fas receptor (*Fas*), NKp46 (Ncr1), perforin (*Prf1*), *GzmB*, natural killer cell factor 1.1 (NK1.1, *Klrb1c*), NK chemoattractant CXCL14 (*Cxcl14*), NK cell receptor NKG2D (*Klrk1*), macrophage marker CD68 (*Cd68*), dendritic cell marker CD74 (*Cd74*), neutrophil marker Gr1 (*Ly6g*; previously designated Gr1/Ly6g), and endothelial cell marker CD31 (*Pecam1*).

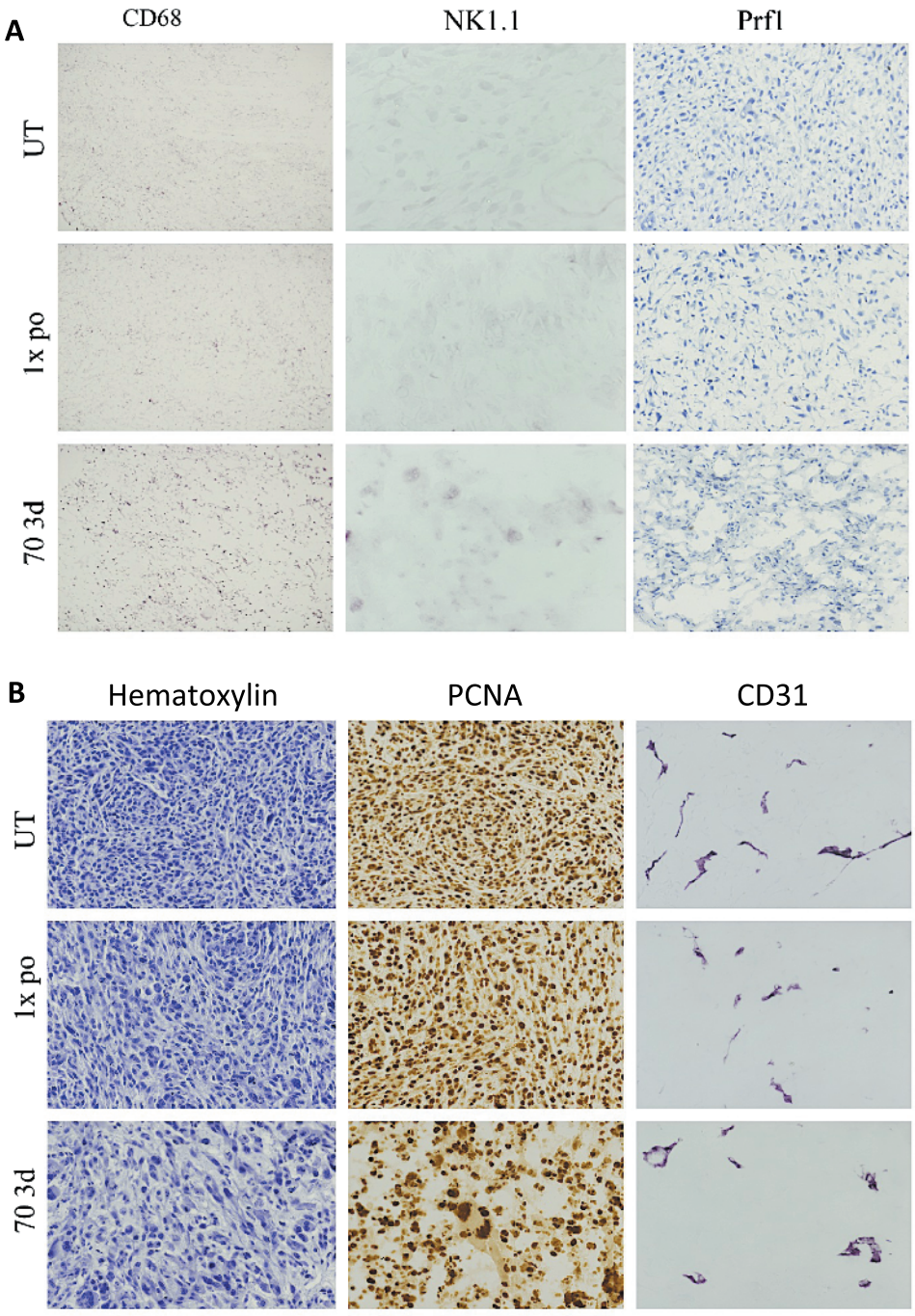

Figure W1. Immunostaining and hematoxylin staining of 9L tumors. (A) Immunostaining of macrophage marker CD68 (original magnification, ×4.2), NK cell marker NK1.1 (×40), and NK cell effector Prf1 (×20) in untreated 9L tumors (UT), or 9L tumors treated with CPA through drinking water (1× p.o.) or every 3 days (70 mg/kg per i.p. injection). Tumors were analyzed on treatment day 24, as in Figure 2*A*. (B) Hematoxylin staining (showing cell density), PCNA staining (showing cell viability), and immunostaining of endothelial cell marker CD31 (all at ×20 magnification) for the same tumor groups shown in A.

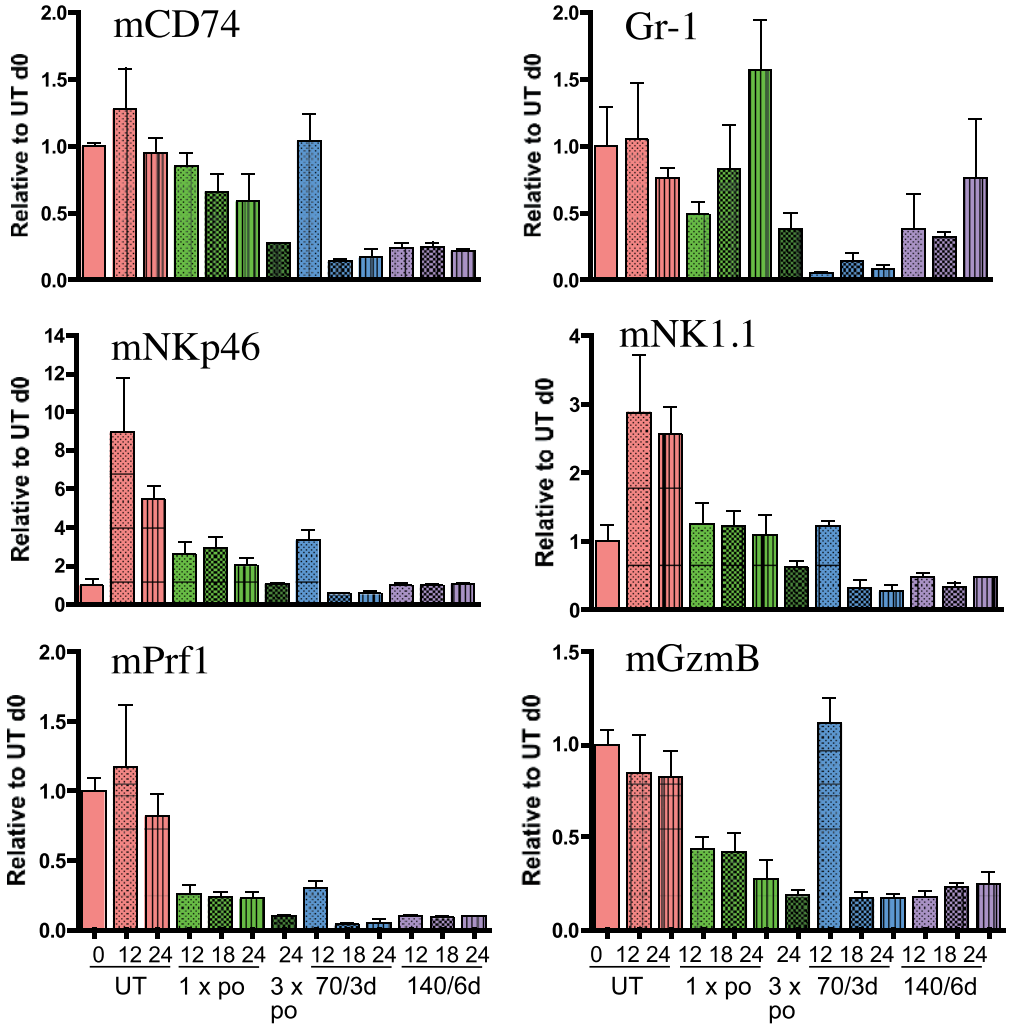

Figure W2. Expression of immune cell markers in spleens of 9L tumor-bearing SCID mice. Mice were untreated (UT) or were treated with various metronomic CPA regimens: oral, 23.3 mg/kg BW daily (1× po); oral, 70 mg/kg BW daily (3× po); i.p., 70 mg/kg BW every 3 days (70/3 days); or i.p., 140 mg/kg every 6 days (140/6 days). Spleens were removed and analyzed on treatment days 0, 12, 18, or 24, as marked. qPCR primers were specific for host (m, mouse) genes, including neutrophil marker Gr1, as in Figures 1*D*, 3, *B* and *C*, 4, *B* and *D*, and 5*B*. Error bars, mean ± SD for $n = 3$ replicate assays of pooled samples from spleens collected from $n = 2$ to 3 mice per treatment group.

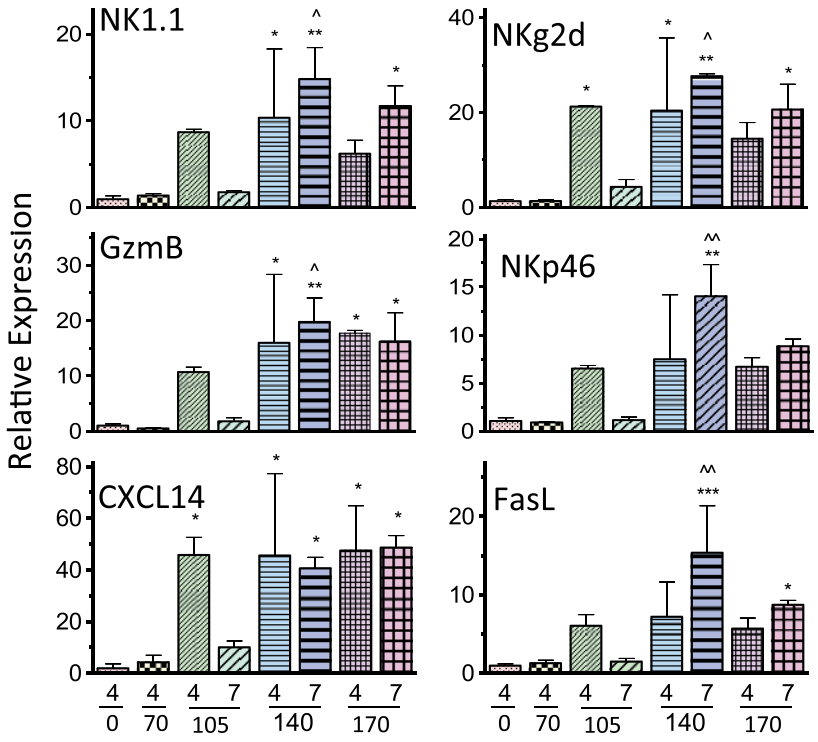

Figure W3. Expression of the indicated immune cell and chemokine markers in metronomic CPA-treated 9L tumors. Tumor RNA samples were assayed on day 6 after either four or seven metronomic CPA cycles, corresponding to treatment days 24 and 42 in Figure 4*A*. Error bars, mean ± SE for $n = 2$ to 4 mice per treatment group. *, **, ***$P < .05$, .01, and .001, respectively, *versus* UT (CPA dose of 0 mg/kg BW). ^, ^^$P < .05$ and .01, respectively, *versus* CPA dose of 105 mg/kg BW treatment on day 6 after seven cycles of CPA treatment. Data were analyzed by one-way ANOVA with Bonferroni multiple comparison correction for one tumor randomly selected from each mouse.

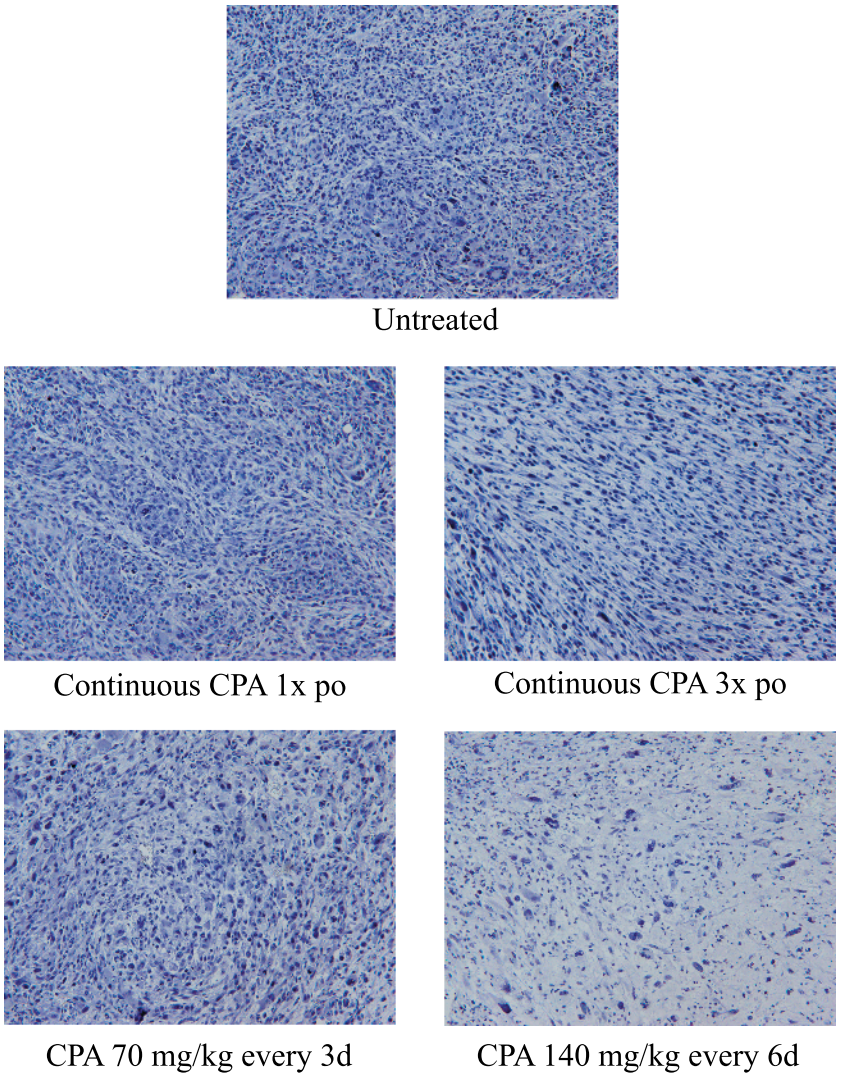

Figure W4. 9L tumor hematoxylin staining. The figure shows relative 9L tumor cell density and morphologic changes in response to 6-day metronomic CPA treatment at doses of 70, 105, 140, or 170 mg/kg. Tumors excised on treatment day 24 (6 days after the fourth cycle of CPA/6-day treatment). Images shown are representative of $n = 10$ to 40 photos taken for each of $n = 2$ to 3 tumors randomly selected from $n = 2$ to 3 mice per treatment group.

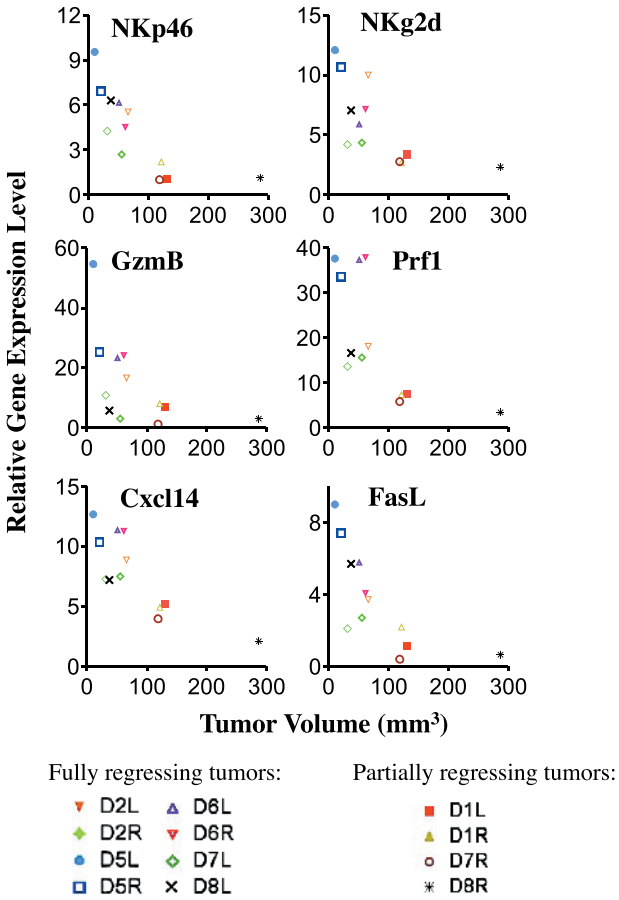

Figure W5. Inverse relationship between tumor volume and expression level of innate immune-related markers. Data are from 9L tumors treated with metronomic CPA at 140 mg/kg per 6 days for 24 days. Data presented in Figure 5 are regraphed to highlight the inverse relationship between tumor volume and relative level of tumor expression of the indicated six mouse genes on day 6 after either 8 or 10 cycles of CPA treatment. Each data point represents one of the 12 individual tumors included in this study (Figure 5), as shown at the bottom, where the eight fully regressing tumors (final tumor volume < 100 mm^3, as in Figure 5*B*) and the four partially regressing tumors (final tumor volume > 100 mm^3, as in Figure 5*A*) are identified by tumor number. For mice D7 and D8, one tumor was in the full regression group (tumors D7L and D8L, respectively) and one tumor was not (tumors D7R and D8R, respectively).